

P.a.	TTG	ACT	AAG	CCA	GCC	ACT	ACG	CCA	GCC	ATT	CTG	GCC	CTT	GCC
E.c.	---	-T-	---	T--	--G					C-A	T--	-TT	--G	-AA
S.t.	---	-T-	---	T--	--G					C-A	T--	-TT	--G	-AA

P.a.	GAT	GGC	AGC	ATC	TTT	CGC	GGC	GAA	TCC	ATC	GGC	GCC	GAT	GGC
E.c.	--C	--A	-C-	CAG	---	-A-	--T	CGG	G--	--A	--G	--A	ACA	--T
S.t.	--C	--A	-C-	CAT	---	-A-	--T	CGG	G--	--T	--G	--A	ACG	--T

FIG. 2. Alignment of the 5' 84 nucleotides of the *carA* coding region of *P. aeruginosa* (*P.a.* [20]) with the same region in *E. coli* (*E.c.* [12]) and *S. typhimurium* (*S.t.* [8]). The underlined nucleotides are those not represented in the reported protein sequence (20).

ette from the first to the second proline codon, thereby spanning exactly 12 nucleotides. Such unmelted secondary structure surviving the processivity of the translating ribosome was unprecedented, but examples of secondary structure within the span of the ribosome critically influencing translation are emerging (19).

It is particularly intriguing that the best alignment of *carA* open reading frames from *E. coli*, *Salmonella typhimurium*, and *P. aeruginosa* is obtained by disregarding 12 nucleotides in the region of the reported gap (Fig. 2). This alignment produces identity at 30 of 57 bases immediately following the 12-nucleotide stretch (compared with an expected 14 to 15 on the basis of random chance), while a straight alignment (without looping out 12 bases of *P. aeruginosa*) gives identity at only 10 of 57.

Initial experiments were designed to reproduce the original report, before attempting to identify the contributing component(s). With primers based on sequences 25 or 250 nucleotides upstream and 124 nucleotides downstream of the *carA* initiation codon, segments of *carA* were amplified from pSW4 (20) by PCR and cloned into the 5' terminus of the *lacZ* gene

of a derivative of pBR322, between the genetically engineered *Xba*I and *Apa*I sites (15, 18). Resulting constructs were introduced into *E. coli* XL1-Blue (Stratagene). N-terminal protein sequencing of β -galactosidase fusion protein expressed and purified (9) from two of these constructs showed normal continuous translation through the reported gap, and the protein sequence obtained from the longer of these is shown in Fig. 3a. This was a surprise, as previous attempts to reproduce translational irregularities by the creation of convenient reporter gene fusions have been successful for many systems, perhaps most notably gene 60 of phage T4 (6, 19), with technology identical to that described here.

To more closely approximate the wild-type conditions of the reported hop, it was decided to (i) clone the entire *carA* upstream untranslated transcript and (ii) use *P. aeruginosa* PAO1 as the host strain. (There is no known precedent for a prokaryotic gene whose internal translational control depends on more distant sequences.) Therefore, primers based on the sequences 500 nucleotides upstream and 240 nucleotides downstream of the *carA* TTG initiation codon were used to amplify *carA*, resulting in a 746-nucleotide fragment which was cloned into the *Hind*III and *Eco*RI sites of the multiple cloning site of the gram-negative shuttle vector pQF50 (4), creating a *carA-lacZ* translational fusion. Resulting constructs were initially cloned into *E. coli* and transferred by electroporation into *P. aeruginosa* PAO1. Protein sequencing showed that translation of the *carA* mRNA is continuous in *P. aeruginosa* and did not reveal an untranslated region of 12 nucleotides at the reported position (Fig. 3b).

As the limit of resolution of a theoretical secondary sequence is estimated to be 10% of the primary sequence, it was possible that the reported hop occurs in the *carA-lacZ* fusions,

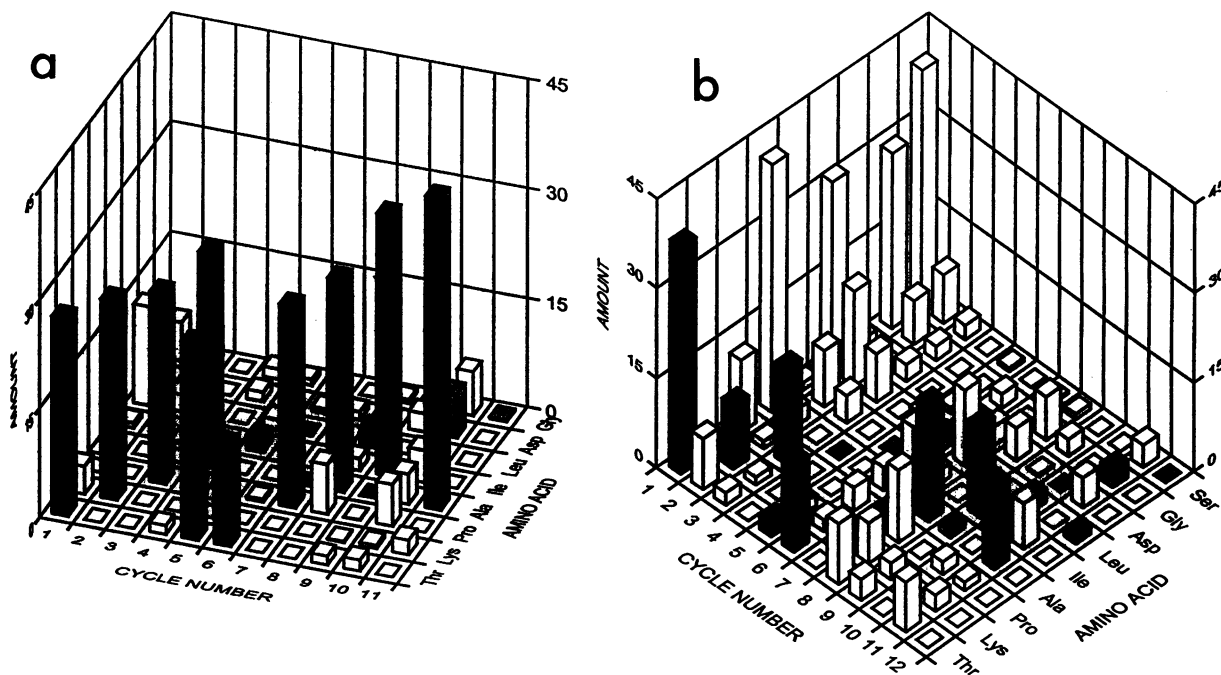


FIG. 3. (a) Graphic representation of N-terminal sequencing of *carA-lacZ* translational fusion which included ~250 nucleotides upstream and ~120 nucleotides downstream of the *carA* initiation codon cloned into a derivative of pBR322 and expressed in *E. coli*. (b) N-terminal sequencing of *carA-lacZ* translational fusion which included ~500 nucleotides upstream and ~240 nucleotides downstream of the *carA* initiation codon, cloned into pQF50, and expressed in *P. aeruginosa*. The heavily shaded bars show the sequence expected from uninterrupted triplet translation, the lightly shaded bars show the sequence expected in the absence of the relevant four amino acids, and the white bars show the background amino acids. Computer graphic representation was obtained by use of the Deltagraph Professional software package (DeltaPoint).

but at a low efficiency. To address this possibility, a construct was designed in which the sixth ACU threonine codon was replaced by a UAA stop codon, by PCR mutagenesis (16), so that any low-level hopping event might be revealed in the absence of normally translated mRNA. On the basis of the sequence of the region, this is the most conservative codon to mutate, as it is the only one which can be deduced to necessarily be included in the unrepresented nucleotides, regardless of which 12-nucleotide span constitutes the gap (Fig. 1a). Expression of the resulting clone failed to produce any measurable β -galactosidase, as judged by enzyme assays, making protein sequence inapplicable. However, as the mechanism of action of the hop in the translation of the native mRNA remains to be understood, the ACU codon could very well be a requirement, and so no positive deductions can be made from the failure of this clone to reveal the hop.

It is possible that a required, *trans*-acting factor is diluted beyond critical concentration for the detection of the bypass, because of the relatively high copy number of the vectors (~30 in *E. coli* for the pBR322-derived constructs and ~13 in *P. aeruginosa* for the pQF50-derived construct). However, there is no *a priori* evidence for such a factor, and its existence is purely speculative.

It is also possible that a *cis*-acting element was not included in the cloning of the gene fusions. This seems unlikely, however, as the entire 500 nucleotides of upstream untranslated sequence and 240 nucleotides of downstream sequence (with respect to the TTG initiation codon) were included in the largest clone. However, there may be something special about the genomic context of *carA*, such that nearby sequences play a role in the hopping event, perhaps through the coupling of transcription and translation.

In spite of the apparent cotranslational hopping over four consecutive codons of the *carA* mRNA in *P. aeruginosa* (20), several attempts to reproduce this phenomenon in *carA-lacZ* fusions have failed. *E. coli* strains were grown in Luria-Bertani broth (tryptone, 10 g/liter; yeast extract, 5 g/liter; NaCl, 5 g/liter), and *P. aeruginosa* was grown in citrate minimal medium (17) at 37°C as reported for the native hop. We can only conclude that unless very special, and subtle, conditions are required, that translation of *carA* of *P. aeruginosa* through the 12 nucleotides of interest proceeds according to the established rules of triplet translation.

We thank A. Abdelal for pSW4, M. Farinha for shuttle vector pQF50, and J. Jesse for *P. aeruginosa* PAO1. We also thank Ed Meenen for expert help with protein sequencing, and Bob Weiss for critical reading of the manuscript.

This work was supported by a research grant from NIH (R01-GM48152) and by the Howard Hughes Medical Institute.

REFERENCES

- Atkins, J. F., R. B. Weiss, and R. F. Gesteland. 1990. Ribosome gymnastics—degree of difficulty 9.5, style 10.0. *Cell* **62**:413–423.
- Benhar, I., and H. Engelberg-Kulka. 1993. Frameshifting in the

- expression of the *E. coli trpR* gene occurs by the bypassing of a segment of its coding sequence. *Cell* **72**:121–130.
- Copper, A. A., and T. H. Stevens. 1993. Protein splicing: excision of intervening sequences at the protein level. *Bioessays* **15**:667–674.
- Farinha, M. A., and A. M. Kropinski. 1990. Construction of broad-host-range plasmid vectors for easy visible selection and analysis of promoters. *J. Bacteriol.* **172**:3496–3499.
- Gesteland, R. F., R. B. Weiss, and J. F. Atkins. 1992. Recoding: reprogrammed genetic coding. *Science* **257**:1640–1641.
- Huang, W. M., S.-W. Ao, S. Casjens, R. Orlandi, R. Zeikus, R. B. Weiss, D. Winge, and M. Fang. 1988. A persistent untranslated sequence within bacteriophage T4 DNA topoisomerase gene 60. *Science* **239**:1005–1012.
- Kane, J. F., B. N. Violand, D. F. Curran, N. R. Staten, K. L. Duffin, and G. Bogosian. 1992. Two codon translational hop during synthesis of bovine placental lactogen in a recombinant strain of *Escherichia coli*. *Nucleic Acids Res.* **20**:6707–6712.
- Kilstrup, M., C. Lu, A. Abdelal, and J. Neuhaard. 1988. Nucleotide sequence of the *carA* gene and regulation of the *carAB* operon in *Salmonella typhimurium*. *Eur. J. Biochem.* **176**:421–429.
- Matsudaira, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **262**:10035–10038.
- Michel, F., A. D. Ellington, S. Couture, and J. W. Szostak. 1990. Phylogenetic and genetic evidence for triple bases in catalytic domain of group I introns. *Nature (London)* **347**:578–580.
- O'Connor, M., R. F. Gesteland, and J. F. Atkins. 1989. tRNA hopping: enhancement by an extended anticodon. *EMBO J.* **8**:4315–4323.
- Piette, J., H. Nyunoya, C. J. Lusty, R. Cunin, G. Weyens, M. Crabeel, D. Charlier, N. Glandsdorff, and A. Piérard. 1984. DNA sequence of the *carA* gene and the control region of *carAB*: tandem promoters, respectively controlled by arginine and the pyrimidines, regulate the synthesis of carbamoyl-phosphate synthetase in *Escherichia coli* K12. *Proc. Natl. Acad. Sci. USA* **81**:4134–4138.
- Puglisi, J. D., J. R. Wyatt, and I. Tinoco, Jr. 1990. Conformation of a pseudoknot. *J. Mol. Biol.* **214**:437–453.
- Roberts, R. W., and D. M. Crothers. 1992. Stability and properties of double and triple helices: dramatic effects of RNA or DNA backbone composition. *Science* **258**:1463–1466.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Shyamala, V., and G. F.-L. Ames. 1991. Use of exonuclease for rapid polymerase-chain-reaction-based in vitro mutagenesis. *Gene* **97**:1–6.
- Stalon, V., F. Ramos, A. Piérard, and J. M. Wiame. 1967. The occurrence of a catabolic and an anabolic ornithine carbamoyl-transferase in *Pseudomonas*. *Biochim. Biophys. Acta* **139**:91–97.
- Weiss, R. B., D. M. Dunn, J. F. Atkins, and R. F. Gesteland. 1987. Slippery runs, shifty stops, backward steps, and forward hops: –2, –1, +1, +2, +5 and +6 ribosomal frameshifting. Cold Spring Harbor Symp. Quant. Biol. **52**:687–693.
- Weiss, R. B., D. M. Dunn, J. F. Atkins, and R. F. Gesteland. 1990. Ribosomal frameshifting from –2 to +50 nucleotides. *Prog. Nucleic Acid Res. Mol. Biol.* **39**:159–183.
- Wong, S. C., and A. T. Abdelal. 1990. Unorthodox expression of an enzyme: evidence for an untranslated region within *carA* from *Pseudomonas aeruginosa*. *J. Bacteriol.* **172**:630–642.